# Tumors Expressing the Cytosine Deaminase Suicide Gene Can Be Eliminated *in Vivo* with 5-Fluorocytosine and Induce Protective Immunity to Wild Type Tumor

Craig A. Mullen,[1] Melissa M. Coale, Robert Lowe, and R. Michael Blaese

*Metabolism Branch, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, NIH, Bethesda, Maryland 20892*

## ABSTRACT

Successful expression of the cytosine deaminase (CD) suicide gene *in vivo* is demonstrated in three weakly immunogenic murine tumor models: the 102 and 205 fibrosarcomas and the 38 adenocarcinoma. Normal mammalian cells do not contain cytosine deaminase, but tumor cells transduced with retroviral vectors containing the CD gene metabolize the relatively nontoxic prodrug 5-fluorocytosine to the highly toxic 5-fluorouracil. *In vitro* cells expressing the CD gene are killed by 5-fluorocytosine while unmodified cells are not. When injected into syngeneic mice, CD+ tumors can also be eliminated *in vivo* by systemic treatment with 5-fluorocytosine without significant toxicity to the host. Animals whose CD+ tumors were eliminated with prodrug treatment resist subsequent rechallenge with unmodified wild type tumor. This posttreatment immunity appears to be tumor specific. Applications of the CD system in gene therapy models are discussed.

## INTRODUCTION

Viruses, bacteria, and fungi have many distinct metabolic pathways not found in mammalian cells. Drugs developed for treatment of infections often target these unique enzyme pathways which metabolize relatively nontoxic prodrugs to highly toxic forms. Transfer of the genes encoding these distinctive enzymes, sometimes called suicide genes, to mammalian cells confers upon the genetically altered cells novel chemosensitivity to prodrugs.

CD[2] is an enzyme found in some bacteria and fungi which deaminates cytosine to uracil (1–3). It also deaminates the relatively nontoxic 5-FC to the highly toxic 5-FU (4). Normal mammalian cells do not contain CD and are relatively resistant to 5-FC (5). We and others have recently shown that transfer of a modified CD gene to mammalian cells renders them selectively sensitive to 5-FC *in vitro* (6, 7). Such a negative selection system could be quite useful in basic science and gene therapy studies if it could also function *in vivo*.

In this report, we demonstrate that tumor cells which have been engineered with retroviral vectors to express the CD gene can be eliminated *in vivo* by systemic treatment of animals with parenteral 5-FC. Animals rendered tumor free by treatment with 5-FC also develop posttreatment immunity to wild type, unmodified tumor.

## MATERIALS AND METHODS

**Cell Lines.** All tumor cell lines were derived from C57BL/6 mice. All lines were cloned prior to gene transduction. 38 is a nonmetastatic dimethylhydrazine-induced colon adenocarcinoma, while 102 and 205 are nonmetastatic, methylcholanthrene-induced fibrosarcomas. As previously described, the tumors are weakly immunogenic (8). PLCD12 and LNL6 are nonmalignant murine fibroblast cell lines which produce retroviral vectors; both were derived from PA317 cells which were ultimately derived from NIH/3T3 TK⁻ cells (9).

PLCD12 expresses genes for cytosine deaminase and NeoR while LNL6 expresses the NeoR gene only. Cells were grown in RPMI supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine.

**Gene Transfer.** Tumor cells were transduced with retroviral vectors as described previously (10). The suffix "CD" indicates the cell was transduced with a vector containing the CD and NeoR genes, while the suffix "CDP" identifies a cell transduced with a vector containing the CD gene and a puromycin resistance gene. Cells were exposed to vector supplemented with protamine (5 μg/ml) overnight and then grown for 2 days in RPMI supplemented with 10% heat-inactivated fetal calf serum. They were then grown in G418 (0.6 mg/ml) for 10–14 days and cloned by limiting dilution before further characterization.

**Enzyme Activity Measurement.** CD enzyme activity was determined as described previously by measuring *in vitro* conversion of tritiated cytosine to uracil by lysates of cells (6). Cytosine and uracil were separated by thin layer chromatography, and radioactivity was quantitated by liquid scintillation. Activity is described as pmol cytosine converted to uracil/$10^6$ cells/min.

**Clonogenic Assays.** Five hundred cells were inoculated into 3.5-cm diameter tissue culture wells and grown for 7–10 days in the presence of 5-FC at concentrations specified in the tables. Cells were then fixed with methanol and stained with Geimsa; macroscopic colonies were counted.

**Tumor Growth and Drug Treatment *in Vivo*.** Animals were inoculated s.c. with tissue culture-derived single cell suspensions of cells in 0.1 ml of tissue culture medium or Hanks' balanced salt solution. 5-FC was dissolved in phosphate-buffered saline (12.5 mg/ml), and mice were treated i.p. with 37.5 mg twice daily for 10 days. Animals were followed until death from tumor growth or for at least 8 weeks following tumor inoculation.

**Statistics.** Standard descriptive statistics, the Student $t$ test, regression analysis, and $\chi^2$ tests were used (11).

## RESULTS

**Tumor Cells Expressing the CD Gene Are Sensitive to 5-FC *in Vitro*.** Three poorly immunogenic murine tumor cell lines were transduced with the CD gene *in vitro*. Following selection in G418 and cloning, CD enzyme activity was measured (Table 1). Southern blots were performed on the 38CD and 102CD lines, which demonstrated a single copy of the CD gene in the cells (data not shown). Cell morphology and growth in normal media were unchanged in cells expressing CD. To learn if CD-expressing cells had been rendered sensitive to 5-FC, clonogenic assays were performed in the presence of this prodrug (Fig. 1). Tumors 38CD and 102CD exhibited markedly reduced survival in 5-FC. 205CD was also inhibited but only at higher 5-FC concentrations. There was a clear dose-response relationship between 5-FC concentration and killing in all three lines. The three cell lines were equally sensitive to 5-fluorouracil *in vitro*; 50% lethal dose was approximately 1 μM 5-FU (data not shown). The relationship between CD activity and 5-FC sensitivity was explored in a panel of 38CD tumor cell lines containing a single copy of the CD gene but which varied in enzyme expression. Regression analysis demonstrated a strong inverse relationship between CD enzyme activity and clonogenic survival in 5-FC. The correlation coefficient for the relationship was 0.92.

**CD-expressing Tumor Cell Lines Sensitive to 5-FC *in Vitro* Can Be Eliminated *in Vivo* by Systemic 5-FC Treatment.** Since some CD+ tumor cells could be killed *in vitro* by 5-FC, it seemed possible that *in vivo* these tumors could be inhibited by systemically adminis-

Table 1 *CD activity in tumor cells transduced with CD vectors*

| Cell line | CD gene | Activity[a] (avg. ± SD) | (n) |
|---|---|---|---|
| 102 | − | 3 | (1) |
| 102CD | + | 1582 ± 7 | (2) |
| 205 | − | 11 ± 2 | (3) |
| 205CD | + | 1221 ± 566 | (2) |
| 38 | − | 40 ± 43 | (2) |
| 38CD | + | 1307 ± 367 | (4) |
| 38CDP | + | 673 | (1) |

[a] pmol cytosine converted to uracil/$10^6$ cells/min. *n*, the number of experiments.

tered 5-FC. This could occur only if the tumor cells continued to express the gene *in vivo*; if sufficiently high 5-FC blood or tissue levels could be attained and sustained; and if such drug levels were not lethally toxic to the animals. To find a potentially effective tolerable dose, mice were injected with $1 \times 10^5$ 205CD cells (approximately 10 times the normally tumorigenic dose of cells); 4 days later, cohorts of mice were treated with various doses of 5-FC. While tumor growth was slower and survival extended with regimens as modest as 5 mg twice a day for 5 days, complete suppression of tumor growth (in at least some treated animals) was not seen until a dose of 37.5 mg twice a day for 5 days was given. Drug treatment was extended to 37.5 mg twice a day for 10 days without deaths due to drug treatment and was the treatment regimen used in the remainder of these studies (data not shown). Further dose escalation was not performed.

Table 2 summarizes the suppression of three different CD+ tumors in animals treated with 5-FC. 5-FC treatment did not reduce the incidence or growth rate of wild type tumor. CD+ tumor incidence was dramatically reduced with all three tumor lines. CD+ tumors which did emerge appeared 2–8 weeks after drug treatment and grew more slowly than untreated tumors. There seemed to be a correlation of *in vivo* tumor suppression with sensitivity to 5-FC in clonogenic assays (described above) in that 205CD exhibited greater resistance to 5-FC *in vitro* and *in vivo*.

Delayed 5-FC treatment was less effective in eliminating CD+ tumors. Fig. 2 summarizes the growth of 38CD tumors in mice receiving a single 10-day course of 5-FC initiated on days 4, 11, or 18 after tumor inoculation. As expected, all untreated mice grew tumors; and no animals treated at day 4 grew tumors. Three of five mice treated at 11 days and five of five treated at 18 days grew tumors. However, the growth curves indicate that while delayed drug treatment did not completely eliminate tumors, it did significantly retard tumor growth.

CD enzyme activity was measured in tumors which grew in animals which had received 5-FC (Fig. 3). Significant CD activity was detected in four of five of the 38CD reisolates and four of four 102CD reisolates after 1 month of growth *in vivo*, while almost none was present in three of three 205CD reisolates examined. However, in all cases, the enzyme activity was less than in the original tumor line. Interestingly, reduction of enzyme activity was also seen in 38CD tumors which were grown for 1 month in normal immunocompetent animals not receiving 5-FC.

Growth of distant wild type tumor was unaffected by injection of CD+ tumor cells and systemic treatment with 5-FC. Animals which were injected with tumorigenic doses of wild type tumor on one flank and with CD+ tumor cells on the contralateral flank were treated with 5-FC. The wild type tumors grew normally while the CD+ tumors did not grow (data not shown). This was expected since the toxic 5-FU was produced intracellularly and was not likely to achieve significant systemic concentrations.

**Mice Pretreated with CD+ Tumors and 5-FC Resist Subsequent Challenge with Wild Type Tumor.** To learn if mice which had rejected CD+ tumors after 5-FC treatment had developed immunity to wild type tumor, animals were rechallenged with a tumorigenic dose

of unmodified tumor cells. In all three lines, animals pretreated with CD+ tumor and 5-FC exhibited significant resistance to wild type tumor (Table 3). There appeared to be tumor specificity to the protection in that pretreatment with 102CD but not 38CD was effective in providing protection against wild type 102.

**Immunogenicity of CD.** Because CD is a bacterial protein, it is possible that it would be immunogenic. The decreased tumorigenicity of 102CD cells and the reduced CD activity in 38CD tumors passaged through normal mice would be consistent with that possibility. To more precisely test this, mice were immunized with NIH-3T3-derived syngeneic, nonmalignant vector producer cells which expressed NeoR only (LNL6) or both CD and NeoR genes (PLCD12). They were challenged with either wild type or CD+ tumor cells (Table 4). 38CDP cells were rejected by animals immunized with these unrelated nonmalignant CD+ cells but not by normal mice or animals immunized with cells expressing only NeoR. (38CDP expresses CD and a puromycin resistance gene; it does not contain a NeoR gene.) Immunization with PLCD12 afforded no protection against unmodified 38 tumor cells.

## DISCUSSION

Recently, we reported the modification and expression of the *Escherichia coli* cytosine deaminase gene in mammalian cells and the sensitivity of such CD gene-modified cells to 5-FC *in vitro* (6). The experiments summarized here demonstrate that the CD gene can also function as a negative selection system or suicide gene *in vivo*. The system could be used in a wide variety of settings such as homologous recombination studies (12), safety systems for gene therapy (13), and gene therapy of cancer (14–16) in a manner similar to the *HSV-1-TK* gene. Others have attempted to use CD nongenetically by producing CD immunoconjugates targeted to tumor antigens, thereby attempting to confer tumor-specific drug sensitivity (17).

The sensitivity of tumors to 5-FC was related to enzyme activity, 5-FC dose, and time of treatment. Tumor cells escaped killing if either the levels of CD gene expression were low or the dose or duration of 5-FC treatment were inadequate; thus, successful application of the system *in vivo* will require maximization of intracellular expression of the enzyme as well as effective prodrug delivery. In these experiments, the CD gene was under the influence of the constitutive Moloney murine leukemia virus long terminal repeat, a type of general purpose viral promoter (18). Depending on the potential application, it is conceivable that tissue-specific promoters and/or enhancer elements would be more effective. This is especially true for use of the CD system in nonneoplastic tissue since it has been observed that general purpose viral promoters, while effective *in vitro*, sometimes fail to express genes under their influence once cells are transferred back into a syngeneic host (19). 5-FC delivery *in vivo* will also be critical in successful application of the system. *In vitro* death of cells sensitive to 5-FC seems to require continuous exposure to drug for at least 5 days. Another group studying 5-FC pharmacokinetics in nude mice bearing a CD+ human tumor xenograft recently reported that bolus i.p. injection was more effective than continuous i.v. infusion in suppressing tumor growth (20). However, animals on the bolus protocol were treated for many more days than those in the continuous infusion group. Thus, the duration of drug treatment could be very important for elimination of CD+ cells *in vivo*. This would be consistent with our observation of significant CD enzyme activity in some larger tumors which were initially inhibited but resumed growth after 10 days of drug treatment.

Animals treated with CD+ tumors and 5-FC often exhibited resistance to later challenge with wild type tumor. Among the tumors tested here, there was specificity to the protection, suggesting that the
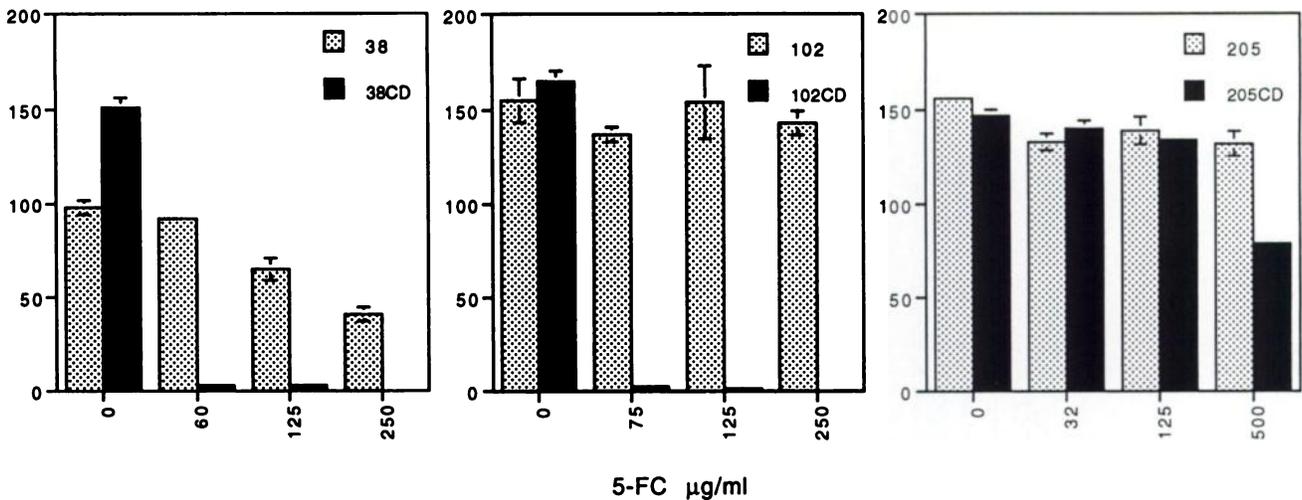
1504

## Colonies



5-FC µg/ml

Fig. 1. Clonogenic growth potential in 5-FC of tumor cells transduced with the CD gene. Five hundred cells were inoculated into each well containing 5-FC at the designated concentration. Colonies were stained and counted 7 days later. Each condition was performed in triplicate. *Bars,* SD; where bars are not visible, SD was less than 2.

Table 2 *CD+ tumors are inhibited in vivo by systemic 5-FC*

| Tumor | Tumor incidence (%)[a] | |
| | No 5-FC | 5-FC |
|---|---|---|
| 102 | 20/20 (100) | 24/24 (100) |
| 102CD | 18/28 (64) | 2/35 (6)[b] |
| | | |
| 205 | 10/10 (100) | 10/10 (100) |
| 205CD | 15/15 (100) | 8/17 (47)[b] |
| | | |
| 38 | 9/9 (100) | 11/11 (100) |
| 38CD | 10/10 (100) | 3/50 (6)[b] |
| 38CDP | 10/10 (100) | 0/10 (0) |

[a] Mice were injected with $1 \times 10^5$ tumor cells. Three to 5 days later, treatment with 5-FC was started as described in "Materials and Methods."

[b] CD+ tumors in 5-FC treated animals emerged 2–8 weeks later than those in mice not treated with 5-FC.
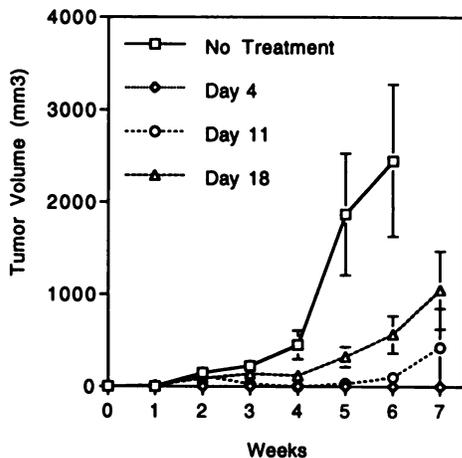


Fig. 2. Effect of delayed 5-FC treatment on the growth of 38CD tumors. Mice received a 10-day course of 5-FC initiated 4, 11, or 18 days after tumor inoculation [average tumor volume (± SE) at various times after tumor inoculation is plotted]. Tumor incidence is described in the text.

effect was T-cell mediated. Several explanations for this effect exist: (*a*) it is possible that elimination of CD+ cells by 5-FC is the metabolic equivalent of surgical tumor amputation, a method which sometimes induces immunity; (*b*) it is possible that the death of CD+ cells *in vivo* leads to more effective antigen presentation. Conceivably, the inflammatory response to the dying cells could recruit and activate

antigen-presenting cells; and (*c*) the CD protein itself seems to be immunogenic. While immunity directed against the CD protein will not lead to direct rejection of wild type tumor which does not express CD, the protein may function as a superantigen of sorts leading to polyclonal activation of lymphocytes, some of which may be cross-reactive with the tumor. As noted above, this immunological effect was not potent enough to cause rejection of bulky preexisting wild type tumor in an animal bearing both wild type and CD+ tumors. However, the later rejection of wild type tumor after immunization with CD+ tumors suggests that the CD suicide gene could be useful in development of autologous tumor vaccines for treatment of minimal residual disease.

Suicide gene systems using the herpes thymidine kinase gene are being used in clinical trials for treatment of brain tumors (16) and for adoptive immunotherapy of human immunodeficiency virus infection (13). In the brain tumor trial cells secreting HSV-1-TK vectors are injected into a tumor bed in an attempt to render the tumor sensitive to the prodrug. CD may be a useful alternative to the HSV-1-TK system. One advantage of CD is that its prodrug, 5-FC, while useful in treatment of some fungal disorders, can be replaced by many alternative agents in the treatment of such infections. Contrarily, the prodrugs for the HSV-1-TK system, ganciclovir and acyclovir, are the only effective agents for the treatment of herpes infections, and their use could result in unwanted, premature elimination of the modified cells in a gene therapy protocol. The availability of alternate suicide

Table 3 *Resistance to wild type tumor exhibited by animals pretreated with 5-FC and tumor cells transduced with the CD gene*

| Tumor challenge[a] | Pretreatment[b] | Tumor incidence (%)[c] | |
|---|---|---|---|
| 38 | None | 15/15 (100) | |
| | 38CD | 6/29 (21) | [d] (P < 0.0001) |
| 102 | None | 15/15 (100) | |
| | 102CD | 12/23 (52) | [d] (P = 0.002) |
| | 38CD | 9/10 (90) | |
| 205 | None | 10/10 (100) | |
| | 205CD | 2/10 (20) | [d] (P < 0.0001) |

[a] Animals were challenged with $10^6$ wild type 38 or 102 cells or $10^4$ 205 cells.

[b] Four to 6 weeks prior to challenge with wild type tumor, mice received $10^6$ 38CD or 102CD cells or $10^4$ 205CD cells, followed by a 2-week course of 5-FC.

[c] Number of animals with tumor per number of animals challenged.

[d] Tumor incidence significantly less than in no pretreatment group; P was calculated from $\chi^2$ analysis.
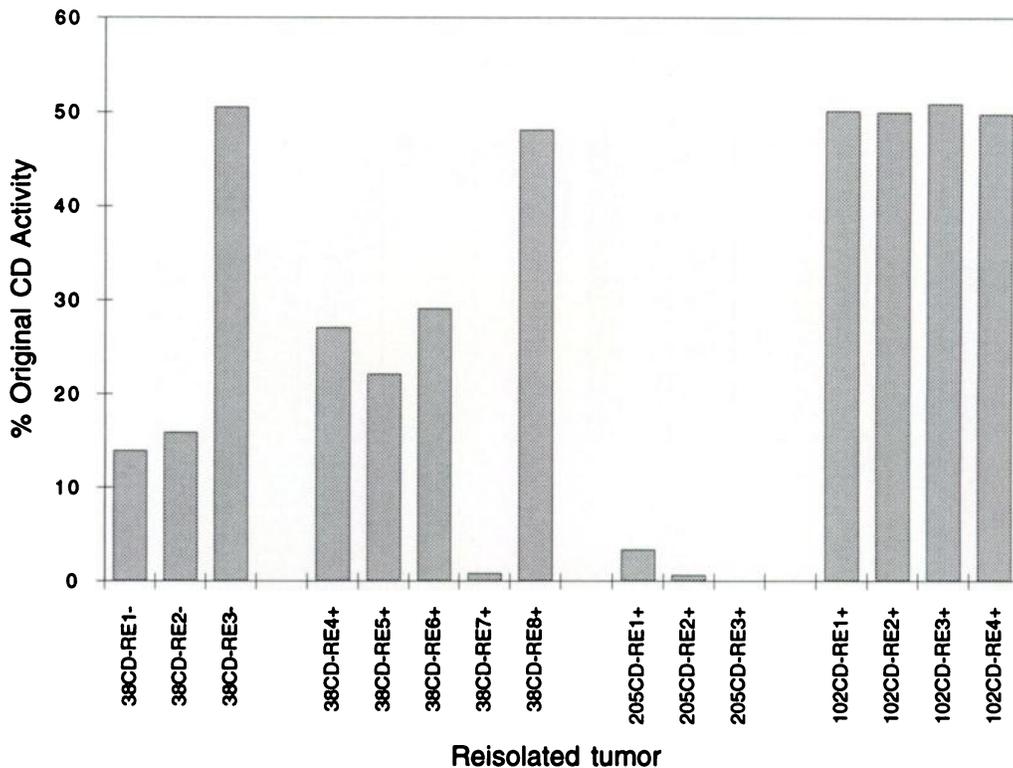
1505

Fig. 3. CD activity in tumor lines after passage *in vivo*. Progressively growing tumors were resected and adapted to tissue culture 1 month after inoculation and 2–3 weeks after 5-FC treatment. (+) indicates the tumor grew in an animal treated with 5-FC while (-) indicates the tumor grew in an untreated animal. Lines 38CD-RE1, 38CD-RE2, and 38CD-RE3 were derived from mice that were not treated with 5-FC. All other mice received drug. Activity is expressed as a percentage of activity in CD+ lines which were initially injected into the mice.

Table 4 *Immunization of mice with unrelated, nonmalignant cells expressing the CD gene protects against rechallenge with CD+ tumor cells*

| Immunizing cell[a] | Gene | Tumor incidence[b] | |
| --- | --- | --- | --- |
| | | 38 | 38CDP |
| None | | 5/5 | 8/10 |
| PLCD12 | CD & NeoR | 5/5 | 1/10[c] |
| LNL6 | NeoR | 5/5 | 5/5 |

[a] Mice were immunized with $1 \times 10^6$ cells 2 weeks before challenge with tumor.

[b] Mice were inoculated s.c. with $1 \times 10^5$ tumor cells. 38CDP tumor cells express the CD and puromycin resistance genes.

[c] Tumor incidence significantly less than in the "None" and "LNL6" groups ($P = 0.001$ by $\chi^2$ analysis). Data are pooled from two experiments.

gene systems such as CD may provide greater flexibility in the gene therapy of cancer and other disorders.

## REFERENCES

1. Danielsen, S., Kilstrup, M., Barilla, K., Jochimsen, B., and Neuhard, J. Characterization of the *Escherichia coli codBA* operon encoding cytosine permease and cytosine deaminase. Mol. Microbiol., *6:* 1335–1344, 1992.
2. Kilstrup, M., Meng, L. M., Neuhard, J., and Nygaard, P. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli.* J. Bacteriol., *171:* 2124–2127, 1989.
3. Andersen, L., Kilstrup, M., and Neuhard, J. Pyrimidine, purine and nitrogen control of cytosine deaminase synthesis in *Escherichia coli* K12. Involvement of the *glnLG* and *purR* genes in the regulation of *codA* expression. Arch. Microbiol., *152:* 115–118, 1989.
4. Polak, A., and Scholer, H. J. Mode of action of 5-fluorocytosine and mechanisms of resistance. Chemotherapy (Basel), *21:* 113–130, 1975.
5. Polak, A., Eschenhof, E., Fernex, M., and Scholer, H. J. Metabolic studies with 5-fluorocytosine-14-C in mouse, rat, rabbit, dog and man. Pharmacology (Basel), *22:* 137–153, 1976.
6. Mullen, C. A., Kilstrup, M., and Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc. Natl. Acad. Sci. USA, *89:* 33–37, 1992.
7. Austin, E. A., and Huber, B. E. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of *Escherichia coli* cytosine deaminase. Mol. Pharmacol., *43:* 380–387, 1993.
8. Restifo, N. P., Esquivel, F., Asher, A. L., Stotter, H., Barth, R. J., Bennick, J. R., Mule, J. J., Yewdell, J. W., and Rosenberg, S. A. Defective presentation of endogenous antigens by a murine sarcoma: implications for the failure of an anti-tumor immune response. J. Immunol., *147:* 1453–1459, 1991.
9. Miller, A. D., and Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol., *6:* 2895–2902, 1986.
10. Cornetta, K., and Anderson, W. F. Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene-transfer: implications for human gene therapy. J. Virol. Methods, *23:* 187–194, 1989.
11. Altman, D. G. Practical Statistics for Medical Research. London: Chapman and Hall, 1991.
12. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (Lond.), *336:* 348–352, 1988.
13. Riddell, S. R., Greenberg, P. D., Overell, R. W., Loughran, T. P., Gilbert, M. J., Lupton, S. D., Agosti, J., Scheeler, S., Coombs, R. W., and Corey, L. Phase 1 study of cellular adoptive immunotherapy using genetically modified CD8+ HIV-specific T-cells for HIV seropositive patients undergoing allogeneic bone marrow transplant. Hum. Gene Ther., *3:* 319–338, 1992.
14. Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Res., *46:* 5276–5281, 1986.
15. Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science (Washington DC), *256:* 1550–1552, 1992.
16. Oldfield, E. H., Ram, Z., Culver, K. W., Blaese, R. M., DeVroom, H. L., and Anderson, W. F. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. Hum. Gene Ther., *4:* 39–69, 1993.
17. Senter, P. D., Su, P. C., Katsuragi, T., Sakai, T., Cosand, W. L., Hellström, I., and Hellström, K. E. Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates. Bioconjug. Chem., *2:* 447–451, 1991.
18. Miller, A. D., and Rosman, G. J. Improved retroviral vectors for gene transfer and expression. Biotechniques, *7:* 980–990, 1989.
19. Palmer, T. D., Rosman, G. J., Osborne, W. R. A., and Miller, A. D. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. Proc. Natl. Acad. Sci. USA, *88:* 1330–1334, 1991.
20. Huber, B. E., Austin, E. A., Good, S. S., Knick, V. C., Tibbels, S., and Richards, C. A. *In vivo* antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res., *53:* 4619–4626, 1993.

# Tumors Expressing the Cytosine Deaminase Suicide Gene Can Be Eliminated *in Vivo* with 5-Fluorocytosine and Induce Protective Immunity to Wild Type Tumor

Craig A. Mullen, Melissa M. Coale, Robert Lowe, et al.

| | |
|---|---|
| **Updated version** | Access the most recent version of this article at:<br>http://cancerres.aacrjournals.org/content/54/6/1503 |

<br>

| | |
|---|---|
| **E-mail alerts** | Sign up to receive free email-alerts related to this article or journal. |
| **Reprints and Subscriptions** | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| **Permissions** | To request permission to re-use all or part of this article, use this link<br>http://cancerres.aacrjournals.org/content/54/6/1503.<br>Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)<br>Rightslink site. |